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Heterogeneity in P-glycoprotein (multidrug resistance) activity among murine peripheral T cells: correlation with surface phenotype and effector function

P-glycoprotein (P-gly) is the transmembrane efflux pump responsible for multidrug resistance in tumor cells. Functional P-gly activity can be conveniently assessed microfluorometrically using the fluorescent dye rhodamine 123 (Rh123), which is an artificial substrate for the P-gly transporter. Here we assess P-gly activity in subsets of mouse peripheral T lymphocytes using the Rh123 efflux assay. Our data indicate that virtually all $CD8^+$ cells extrude Rh123 efficiently, whereas only a subset of $CD4^+$ cells exhibit P-gly activity. Correlation of P-gly activity in $CD4^+$ cells with the expression of a panel of surface markers revealed that cells bearing an "activated/memory" phenotype ($CD45RB^-$, $CD44^{hi}$, $CD62L^-$, $CD25^+$, $CD69^+$) were exclusively found in the fraction that can extrude Rh123. In contrast "naive" phenotype $CD4^+$ cells ($CD45RB^+$, $CD44^{lo}$, $CD62L^+$, $CD25^-$, $CD69^-$) could be further subdivided into two major subsets based on P-gly activity. In functional studies of sorted cell populations the Rh123-extruding subset of "naive" $CD4^+$ cells proliferated more strongly and secreted higher levels of interleukin (IL)-2 than its Rh123-retaining counterpart when activated by a variety of polyclonal stimuli. Furthermore, this subset produced detectable levels of interferon (IFN)-γ upon stimulation but no IL-4 or IL-10. As expected, the Rh123-retaining "naive" subset produced only IL-2 after stimulation, whereas the "memory" subset produced IFN-γ, IL-4 and IL-10 in addition to low levels of IL-2. Collectively, our data indicate that P-gly activity is a novel parameter that can be used to distinguish a subset of "preactivated" $CD4^+$ cells that would be considered as naive on the basis of their surface phenotype.

1 Introduction

Progress in our understanding of T cell activation and memory has been greatly facilitated by the development of mAb recognizing surface glycoproteins that are differentially expressed by resting and activated T cells [1–6]. For instance, peripheral CD4 T cells in several species can be classified into naive or memory populations by their differential expression of a number of markers including CD44, CD45 and CD62L (L-selectin). In the mouse naive T cells are in the $CD44^{lo}$ $CD45RB^+$, $CD62L^+$ CD4 subset that gradually disappears after adult thymectomy, while memory cells are found within the $CD44^{hi}$, $CD45RB^-$ $CD62L^-$ population [7–9]). These phenotypic profiles have been shown to correlate with specific cytokine-secretion patterns [10–13]. In general, naive CD4 T cells produce mainly IL-2 upon stimulation, whereas memory cells secrete several other cytokines, including IFN-γ and IL-4, in addition to IL-2.

Another surface glycoprotein that seems to be differentially expressed in T cells is P-glycoprotein (P-gly), the energy-dependent transmembrane pump that mediates efflux of a diverse group of lipophilic compounds [14]. P-gly activity is responsible for multidrug resistance in tumor cell lines and human tumors *in vivo* [15–17], but several normal tissues as well as inflammatory cells and normal lymphocytes display functional activity associated with P-gly expression [18–23]. In addition, the reduced staining of certain hematopoietic cells with the fluorescent dye rhodamine 123 (Rh123) has been shown to be due to dye efflux mediated by P-gly [24, 25], thus providing a sensitive and convenient flow cytofluorometric assay for P-gly activity. In this report we used the Rh123 efflux assay for a detailed analysis of P-gly activity in subsets of mouse peripheral T cells. In agreement with other recent studies [18–23], our data show that P-gly activity is differentially expressed in $CD4^+$ and $CD8^+$ T cells. Interestingly, within the $CD4^+$ T cell subset, P-gly activity further subdivides "naive" phenotype cells into two subpopulations that vary with respect to proliferation in response to various stimulatory agents and release of cytokines. Collectively, our data suggest that P-gly activity is a novel parameter that can be used to separate truly naive $CD4^+$ cells from functionally "pre-activated" cells that would be considered as naive by other phenotypic criteria.

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Abbreviations: **Cs A:** Cyclosporin A **HPRT:** Hypoxanthine-guanine-phosphoribosyltransferase **IO:** Ionomycin **P-gly:** P-glycoprotein **$Rh123^-$ cells:** Cells that extrude rhodamine 123 **$Rh123^+$ cells:** Cells that retain rhodamine 123 **SEB:** Staphylococcal enterotoxin B

Key words: CD4 subsets / P-glycoprotein / Multidrug resistance

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2 Materials and methods

2.1 Mice

BALB/c and C57BL/6 female mice were obtained from Harlan Olac LTD (Bicester, GB) and maintained in the animal facility of the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. Mls-1^a (Mtv-7) congenic BALB/c (BALB.D2) mice [26] were originally obtained from H. Festenstein (London Hospital Medical College). Unless otherwise stated mice used for experiments were 2–3 months of age.

2.2 Rh123 staining and flow microfluorometry

Lymph node or splenic cells (1×10^6/ml) from BALB/c mice were incubated for 15 min at 37 °C in DMEM containing 0.33 μg/ml of the fluorescent dye Rh123 (Sigma, St. Louis, MO), washed thereafter once and resuspended in dye-free medium. Cells were then normally incubated at 37 °C for at least 3 h to allow maximal dye efflux (in some cases this incubation time was varied as indicated). In some experiments the P-gly inhibitors cyclosporin A (CsA, Sandoz, Basel, Switzerland), verapamil (Sigma) or PSC833 (a kind gift of Dr. D. Roemer, Sandoz) were added during the efflux period. Thereafter cells were washed again and kept at 4 °C.

For double- or triple-fluorescence analyses Rh123-stained cells were treated with a combination of the following mAb: PE-conjugated (Becton Dickinson, Mountain View, CA) or biotinylated GK1.5 (anti-CD4), biotinylated 53-6.7 (anti-CD8), biotinylated 37.51 (anti-CD28, Pharmingen, San Diego, CA), biotinylated IM781 (anti-CD44), biotinylated Mel-14 (anti-CD62L), biotinylated AMT13 (anti-IL-2Rα, Gibco BRL, Basel, Switzerland), biotinylated H1.2F3 (anti-CD69), and PE-conjugated 23G2 (anti-CD45RB, Pharmingen). Streptavidin-Tri-Color (Caltag, San Francisco, CA) was used to reveal biotinylated mAb. Samples were analyzed using a FACScan and the Lysis II Sorfware program (Becton Dickinson). Rh123 fluorescence was always acquired through the FL1 (FITC) channel. In some experiments purified CD4$^+$ cells were isolated by magnetic separation with a MACS column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) [27] prior to Rh123 staining and three-color immunofluorescence analysis.

2.3 Cell sorting

Splenic cells from BALB/c mice were enriched for CD4$^+$ T cells by nylon wool column followed by treatment with IgM anti-CD8 mAb (3.168) plus rabbit complement. Remaining cells were stained with Rh123 according to the protocol given above. After Rh123 efflux cells were labeled with biotinylated anti-CD4 and PE-conjugated anti-CD45RB (23G2) mAb, followed by Streptavidin-Tri-Color. CD45RB$^+$ cells that extruded Rh123 (Rh123$^-$), CD45RB$^+$ cells that retained Rh123 (Rh123$^+$) and CD45RB$^-$ Rh123$^-$ cells were sorted on a FACStar plus (Becton Dickinson) with gates set on live CD4$^+$ cells. The purity of the sorted cell populations (assessed by post-sort analysis) was greater than 98 % in all experiments.

2.4 Cell culture

Cell cultures were routinely maintained in DMEM supplemented with 5 % FBS and 5×10^{-5} M 2-ME. Sorted CD4$^+$ subpopulations from BALB/c mice were stimulated with either concanavalin A (Con A, 2.5 μg/ml), PMA (10 ng/ml) plus ionomycin (IO, 250 ng/ml), staphylococcal enterotoxin B (SEB, 15 μg/ml, Toxin Technology Saratoga, FL) purified anti-CD3 mAb 145-2C11 (coated to wells at various concentrations for 16 h at 4 °C) or allogeneic (C57BL/6, H2^b) T cell-depleted irradiated (3000 rad) splenic stimulator cells (2×10^5). Mls-1^a (BALB.D2) T cell-depleted stimulator cells (2×10^5) were irradiated at 1000 rad. Sorted CD4$^+$ subpopulations were cultured at 5×10^5/ml in microculture wells (200 μl/well), and harvested or pulsed (0.5 μCi [^{3}H] thymidine/well) at the indicated time points. Radioactivity was measured in a liquid scintillation β-counter and data are presented as the mean cpm of duplicate or triplicate microcultures.

2.5 PCR

Total RNA from stimulated cultures of sorted CD4$^+$ subpopulations ($1 \times 10^5 - 2 \times 10^5$ cells) was extracted using the guanidinium isothyocyanate-acid-phenol procedure [28] and suspended in 30 μl H_2O. The reverse-transcription reaction was performed essentially as described by Sambrook et al. [29]. Briefly, RNA aliquots (one fifth of total RNA) were incubated for 5 min at 65 °C with 0.1 μg oligo d(T)$_{12-18}$ (Pharmacia, Uppsala, Sweden) and further incubated for 60 min at 42 °C with 20 U RNasin (Pharmacia), 0.5 mM dNTP, 12 U AMV reverse transcriptase (Boehringer Mannheim, Germany) and reverse transcriptase buffer (60 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM Tris-HCl; pH 8.4) in a final volume of 20 μl. PCR [30] was performed using aliquots (one tenth) of the resulting cDNA. To this was added 200 μM dNTP, 0.5 μg RNase A, 2.5 U Taq polymerase (Ampli Taq, Perkin Elmer Corporation, Pomona, CA) and 0.6 μM of each sense/antisense primer in a total volume of 40 μl containing 1 × PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl; pH 8.4). The conditions for PCR were 1 min at 92 °C, 1 min at 55 °C, and 1 min at 72 °C for 45 cycles using a DNA thermal cycler (Perkin Elmer Corporation). The PCR products were size fractionated in 2 % agarose gels stained with ethidium bromide. BglI-HinfI-digested pBR 328 DNA was used as size marker.

The primers were chosen from different exons and were as follows: IL-2 sense 5′-ATGTACAGCATGCAGCTCGCATC and antisense 5′-GGCTTGTTGAGATGATGCTTTGACA (amplified fragment 502 bp), IL-5 sense 5′-CGCTCACCGAGCTCTGTTGACAAG and antisense 5′-AGCCTTCCATTGCCCACTCTGTAC (amplified fragment 290 bp), IL-6 sense 5′-ATGAAGTTCCTCTCTGCAAGAGACT and antisense 5′-CACTAGGTTTGCCGAGTAGATCTC (amplified fragment 638 bp), IL-10 sense 5′-CAAAGCAGCCTTGCAGAAAAGAG and antisense 5′-AGATCCCTGGATCAGATTTAGAGA (amplified fragment 644 bp), IFN-γ sense 5′-TGAACGCTACACACTGCATCTTGG and antisense 5′-CGACTCCTTTTCCGCTTCCTGAG (amplified fragment 460 bp), hypoxanthine-guanine-phosphoribosyltransferase (HPRT) sense 5′-GTAATGATCAGTCAACGGGGGAC and antisense 5′-CC-

AGCAAGCTTGCAACCTTAACCA (amplified fragment 214 bp).

2.6 Lymphokine assays

IL-2 was quantified using an IL-2-dependent (IL-4 non-responsive) subline of CTLL-2 cells [31]. Supernatants were titrated at various concentrations (v/v) into microcultures containing 10^4 CTLL-2 cells in a final volume of 200 µl/well in flat-bottom plates. The cells were cultured for 48 h and pulsed overnight with 0.5 µCi/well [^{3}H] thymidine. IFN-γ was detected using a two-site sandwich ELISA [32]. Units/ml of IL-2 and IFN-γ were calculated by comparing values of test samples with a standard curve of human rIL-2 or murine rIFN-γ, respectively.

3 Results

3.1 Rh123 efflux as a measure of functional P-gly activity

It has recently been shown that efflux of the fluorescent dye Rh123 from pre-labeled hematopoietic cells [25] or leukocytes [18–23] correlates with functional expression of P-gly, the membrane-associated transport protein responsible for multidrug resistance in tumor cells. As shown in Fig. 1A, a proportion (~ 40 %) of Rh123-labeled mouse lymph node cells lose fluorescence when incubated for 3 h at 37 °C. This loss of Rh123 fluorescence is temperature dependent and can be inhibited by the calcium channel blocker verapamil, a known inhibitor of P-gly function in tumor cells [16, 17]. Other well-characterized P-gly inhibitors include CsA and PSC833, a CsA derivative which does not induce the immunosuppressive effects (*e.g.* inhibition of cytokine production) associated with CsA [33, 34]. This latter compound is about 10 or 100 times more effective (on a molar basis) in blocking the efflux of Rh123 in lymphocytes as compared to CsA or verapamil, respectively (Fig. 1B). Since studies of reversal of P-gly-mediated multidrug resistance in tumor cells show the same hierarchy of these three drugs [35, 36], we conclude that Rh123 efflux in lymphocytes reflects primarily P-gly activity.

3.2 P-gly activity in lymphocyte subsets

Since Rh123 efflux from lymph node cells was heterogeneous, we counter-stained these cells with mAb against CD4 or CD8 at various times after Rh123 loading. As shown in Fig. 2, $CD8^+$ cells extruded Rh123 much more efficiently than $CD4^+$ cells. After 3 h at 37 °C, ~ 90 % of $CD8^+$, as opposed to ~ 40 % of $CD4^+$ cells were $Rh123^-$. In addition, the rate of Rh123 efflux was about three times faster in $CD8^+$ cells ($t_{1/2}$ ~ 30 min) than in $CD4^+$ cells ($t_{1/2}$ ~ 90 min). In contrast to T cells, the majority of $CD4^-$ $CD8^-$ LN cells (corresponding primarily to B cells) did not extrude Rh123 efficiently under these conditions (data not shown).

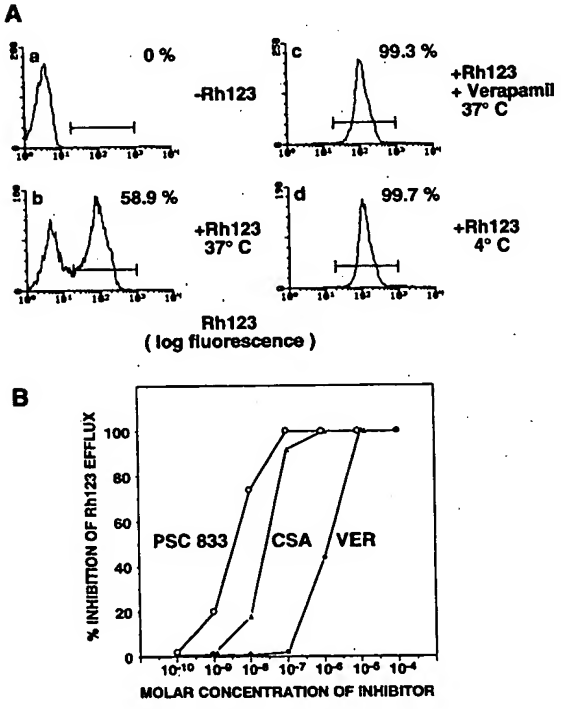

Figure 1. Rh123 efflux in peripheral lymphocytes is blocked by P-gly inhibitors and low temperature. (A) After Rh123 labeling lymph node cells were washed and incubated for 3 h either at 4 °C or at 37 °C in dye-free medium in the presence or absence of verapamil (50 µM). (B) Comparison of P-gly inhibitors PSC833, CsA and verapamil for their ability to block Rh123 efflux in lymph node cells.

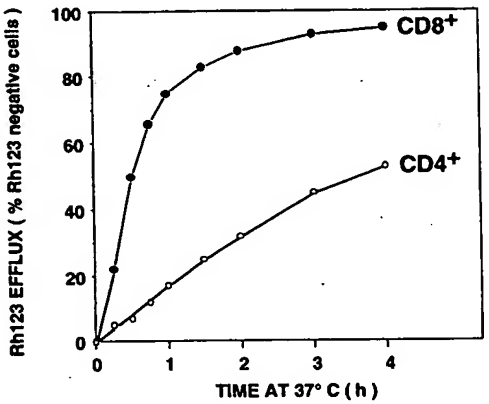

Figure 2. Kinetics of Rh123 efflux in T cell subsets. After Rh123 uptake lymph node cells were washed and incubated at 37 °C in dye-free medium for the time periods indicated and counterstained with anti-CD4 and anti-CD8 mAb. Efflux of Rh123 in individual subsets was measured by gating on $CD4^+$ or $CD8^+$ cells.

3.3 Relationship between P-gly activity and CD45 isoform expression on $CD4^+$ T cells

The differential expression of CD45 isoforms has been used to subdivide mouse $CD4^+$ cell populations into naive- or memory/activated-type cells, the latter being identified by preferential expression of the low molecular weight isoform of CD45 [8, 12]. To investigate the relationship between CD45 isoform expression and P-gly activity among $CD4^+$

peripheral cells, lymph node cells were stained with mAb against CD4 and CD45RB after Rh123 efflux. As shown in Fig. 3, essentially all CD4$^+$ CD45RB$^-$ cells, containing the pool of activated/memory cells, were Rh123$^-$. However, the CD45RB$^+$ (naive) subset of CD4$^+$ cells was heterogeneous, containing both Rh123$^+$ and Rh123$^-$ cells. Thus, there is no strict correlation between P-gly activity and CD45RB expression among CD4$^+$ cells.

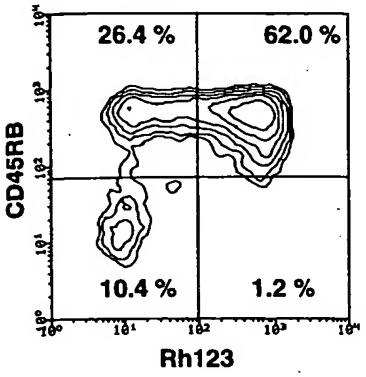

Figure 3. Correlation of Rh123 efflux with CD45RB expression in CD4$^+$ cells. After Rh123 efflux lymph node cells from adult mice were stained with anti-CD4 and anti-CD45RB mAb. Cytogram is gated on CD4$^+$ cells.

3.4 Increase in P-gly function with age

Work in several laboratories has established that aging is accompanied by shifts in the proportions of T lymphocyte subsets in both humans and mice (reviewed in [37]). In general T cells of the naive phenotype are progressively replaced by T cell populations bearing activation/memory markers. In agreement with a recent report [20], the proportion of lymph node as well as splenic CD4$^+$ cells that are Rh123$^-$ increases dramatically in older mice, concomitant with the loss of CD45RB$^+$ cells (Fig. 4). However, as noted above this increase in Rh123$^-$ cells does not strictly correlate with decreased CD45RB expression, since the major increase in Rh123$^-$ cells is found within the CD45RB$^+$ subset.

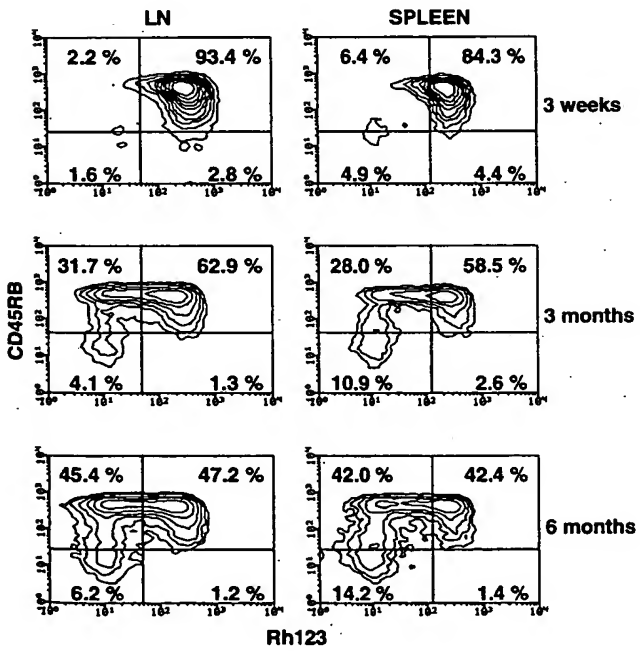

Figure 4. Effect of age on Rh123 efflux and CD45RB expression among CD4$^+$ cells. Lymph node and splenic cells from mice of different ages were stained and analyzed as Fig. 3.

3.5 Relationship between P-gly activity and expression of surface activation markers on CD4$^+$ cells

Activation of resting CD4$^+$ cells is coupled to the selective up-regulation, induction or loss of surface molecules that promote interaction with other cell types, are involved in signaling or allow specific adhesion and migration. To investigate whether P-gly activity correlated with a specific surface phenotype, purified CD4$^+$ cells were allowed to extrude Rh123 and were then counterstained with mAb against CD45RB and a panel of common activation markers including CD62L, CD44, CD69, CD28 and CD25 (Fig. 5). Both the Rh123$^+$ and Rh123$^-$ cells within the CD45RB$^+$ subset showed a similar "naive" phenotype (CD25$^-$, CD69$^-$, CD44^{lo}, CD62L$^+$). In contrast, in the CD45RB$^-$ (Rh123$^-$) population about 50 % of the cells were positive for CD25 or CD69 expression and negative for CD62L. These cells also showed higher surface levels of CD44 as compared to CD45RB$^+$ cells. Thus, CD45RB$^+$ CD4$^+$ cells that differ in P-gly function are otherwise quite homogeneous in phenotype, whereas CD45RB$^-$ CD4$^+$ cells are heterogeneous with respect to several activation/adhesion markers.

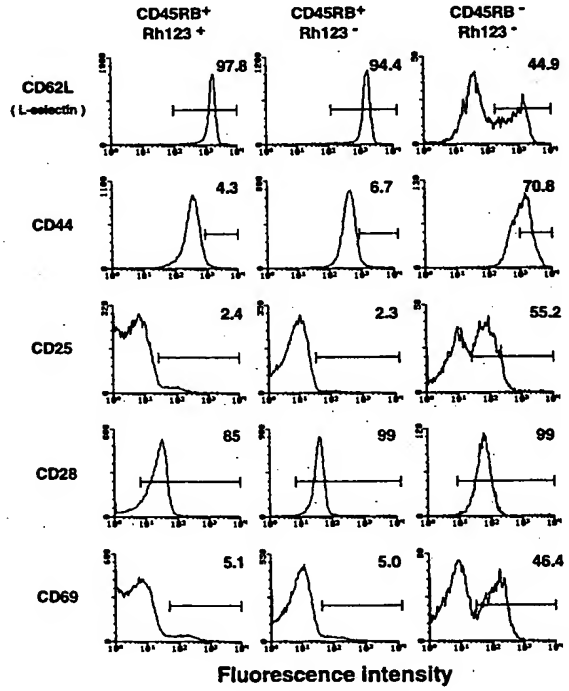

Figure 5. Phenotypic analysis of Rh123$^-$ and Rh123$^+$ CD4$^+$ lymph node cells. After purification by magnetic cell sorting CD4$^+$ cells (99.8 % pure) were allowed to pump out Rh123 and were then counterstained with anti-CD45RB mAb and mAb directed against each of the indicated activation/adhesion markers. Data are presented as histograms gated on CD45RB and Rh123 (see Figs. 3 and 4).

3.6 *In vitro* proliferation of sorted CD4+ T cell subsets

Naive and memory cells not only differ by phenotypic criteria but also in their activation requirements, proliferative responses, secreted cytokine profiles, life-spans and migration patterns [1–6, 38–40]. As mentioned above, the two subpopulations (Rh123$^-$ or Rh123$^+$) of CD45RB$^+$ CD4$^+$ cells were not distinguishable using a number of surface markers. However, the increase in Rh123$^-$ CD4$^+$ cells with age suggested that P-gly activity might be correlated with a previous encounter with environmental antigens. For this reason we compared the *in vitro* proliferative responses of these subpopulations to various stimulatory agents. Fig. 6 summarizes the results of such experiments for sorted CD45RB$^+$ Rh123$^-$, CD45RB$^+$ Rh123$^+$ or CD45RB$^-$ Rh123$^-$ CD4$^+$ cells. Following stimulation with either the plant lectin Con A, the bacterial superantigen SEB, or the pharmacological agents PMA plus IO, the CD45RB$^+$ Rh123$^-$ CD4$^+$ T cell population consistently proliferated most strongly. CD45RB$^+$ Rh123$^+$ cells were intermediate in all cases, while CD45RB$^-$ Rh123$^-$ cells proliferated least (or not at all in the case of SEB stimulation). Addition of rIL-2 to the cultures only had marginal effects on the two CD45RB$^+$ subpopulations, while CD45RB$^-$ cells proliferated up to tenfold better, especially in SEB-activated cultures. Addition of IL-2 alone had no growth-promoting effect on CD45RB$^+$ cells and only gave a slight stimulation of CD45RB$^-$ cells.

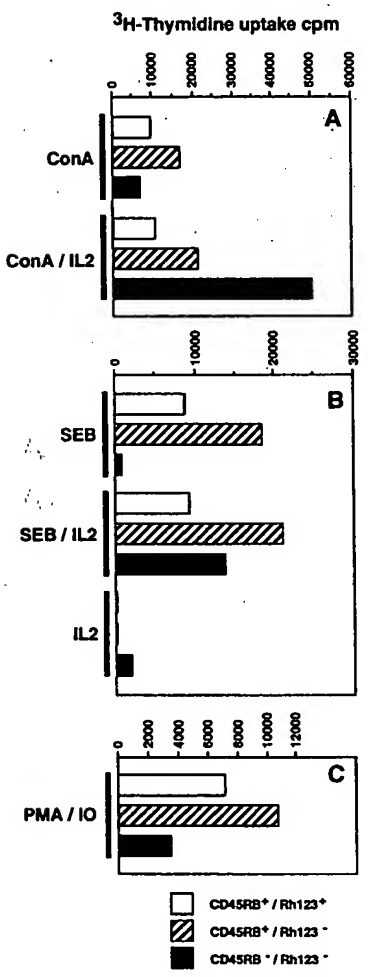

Figure 6. Proliferation of FACS-sorted CD45RB$^+$ Rh123$^-$, CD45RB$^+$ Rh123$^+$ and CD45RB$^-$ Rh123$^-$ CD4$^+$ splenic cells. (A, B) Proliferative response of 1×10^5 cells measured 60 h after stimulation with Con A (A) or SEB (B) in the presence or absence of recombinant IL-2 (50 units/ml). In (C) proliferation of 3×10^4 cells was measured 44 h after induction with PMA and IO. In (A) and (B) cultures were supplemented with T cell depleted, irradiated (3000 rad) accessory cells (2×10^5). Proliferation in the absence of stimulator cells as well as proliferation of stimulators alone was negligible. cpm are given as mean values of triplicate cultures.

3.7 Cytokine production by sorted CD4+ T cell subsets

Naive T cells, memory T cells and various effector T cells produce different types and amounts of lymphokines. In the mouse and human it has been shown that naive T cells secrete primarily IL-2 upon polyclonal activation but within days develop into effectors that can be restimulated to make larger quantities of IL-2 as well as additional lymphokines [41–44]. Memory T cells in addition to IL-2 produce several other lymphokines including IFN-γ and IL-4 [1–6, 10–13].

The high proliferative capacity of the Rh123$^-$ subset of CD45RB$^+$ CD4$^+$ cells (Fig. 6) suggested that this subpopulation might include cells with a "preactivated" functional phenotype. Consistent with this view, this cell population produced up to five times more IL-2 than the CD45RB$^+$ Rh123$^+$ cells, irrespective of the stimulatory agent used (Fig. 7). Using either immobilized anti-CD3 antibodies, PMA plus IO, SEB, or Mls-1^a expressing congenic as well as allogeneic (C57BL/6) stimulator cells, Rh123$^-$ CD45RB$^+$ cells in each case gave the highest IL-2 titers. The stronger IL-2 production of the CD45RB$^+$ Rh123$^-$ compared to the CD45RB$^+$ Rh123$^+$ cells was not due to differing dose response or kinetic requirements of the two subsets (Fig. 7B and Table 1).

Since CD45RB$^+$ Rh123$^-$ cells produced much higher levels of IL-2 than CD45RB$^+$ Rh123$^+$ cells we additionally measured titers for IFN-γ (Fig. 8). Using either anti-CD3 mAb or PMA plus IO as stimulatory agents, IFN-γ could never be detected in the supernatants of CD45RB$^+$ Rh123$^+$ cell cultures, while low amounts of this lymphokine were

Table 1. Kinetics of IL-2 production by stimulated CD45RB$^+$ Rh123$^+$ and Rh123$^-$ subsets of CD4$^+$ T cells[a)]

Stimulus	Time of culture (h)	IL-2 production (U/ml)	
		CD45RB$^+$ Rh123$^+$	CD45RB$^+$ Rh123$^-$
Anti-CD3 mAb	25	2.4	9.1
	41	10.3	36
	67	41	137
PMA + IO	16	12	78
	48	90	198
	72	212	325

a) The indicated FACS-sorted CD4$^+$ subsets (5×10^5 cells/ml) were stimulated with either immobilized anti-CD3 mAb (30 μg/ml) or PMA + IO. Supernatants harvested at the indicated times were assayed for IL-2 activity. Data are expressed as IL-2 U/ml.

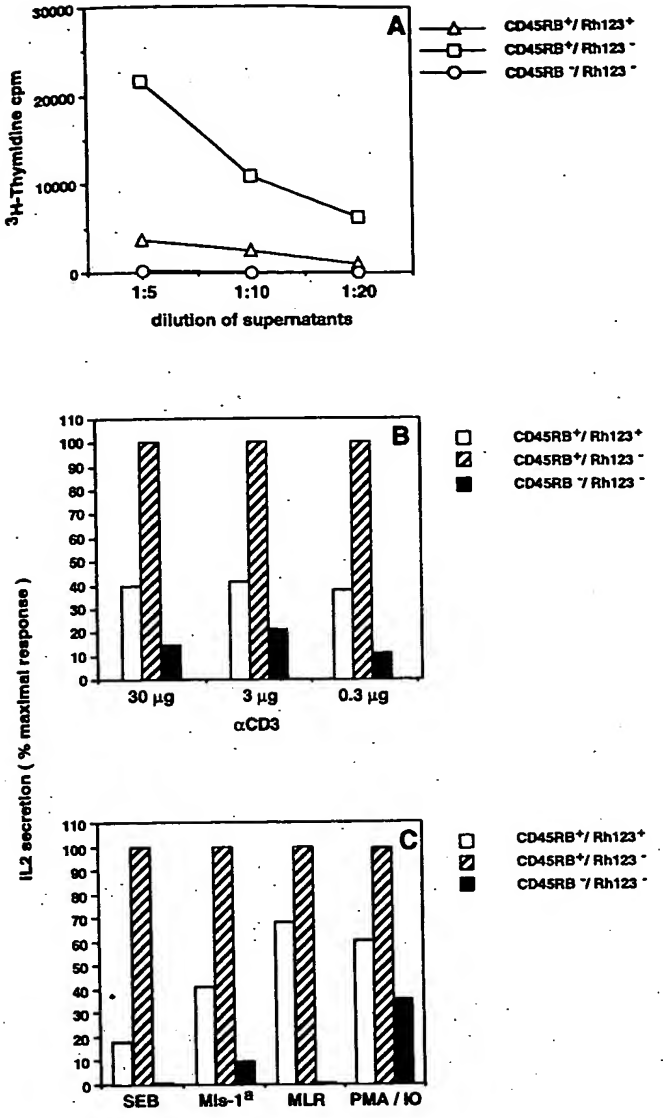

Figure 7. CD45RB$^+$ Rh123$^-$ cells produce enhanced levels of IL-2 under various stimulatory conditions. (A) IL-2 secretion of sorted subpopulations 60 h after induction with SEB as measured by the proliferative response of CTLL-2 cells in the presence of serial dilutions of culture supernatants. (B, C) Comparison of IL-2 secretion among CD4$^+$ subpopulations after stimulation with either immobilized anti-CD3 mAb, PMA plus IO, SEB, congenic BALB.D2 (Mls-1^a) or allogeneic C57BL/6 (MLR) cells. IL-2 secretion is expressed as a percentage of the maximal IL-2 titers observed for each stimulus.

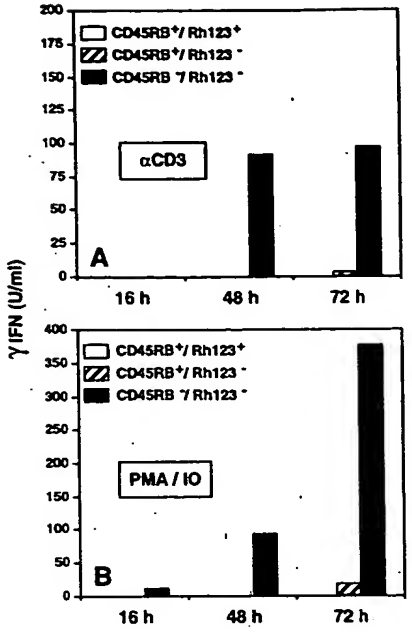

Figure 8. IFN-γ secretion by sorted CD4$^+$ T cell subsets. Sorted CD4$^+$ subpopulations were cultured with immobilized anti-CD3 mAb or PMA plus IO. After 16, 48 and 72 h supernatants were analyzed for IFN-γ by ELISA. Data are representative of three independent expriments.

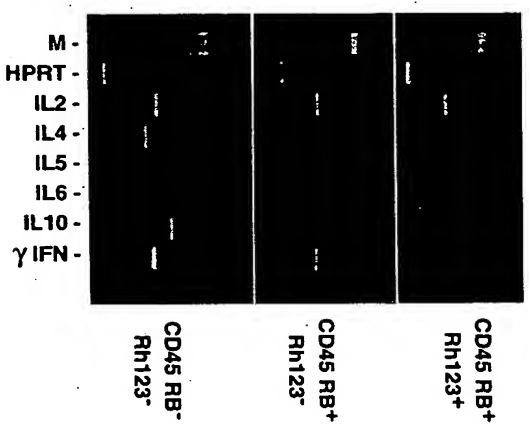

Figure 9. RT-PCR analysis of lymphokine mRNA expression in sorted CD4$^+$ subpopulations 48 h after stimulation with immobilized anti-CD3 mAb. Total RNA was extracted and prepared for RT-PCR analysis as described in Sect. 2.5. PCR amplified products were analyzed by electrophoresis in ethidium bromide-stained 2 % agarose gels. HPRT mRNA expression was used as a control for the integrity of RNA samples. M refers to molecular size markers (in bp: 154, 220/234, 298, 394, 453, 517, 653, 1033, 1230, 1766, 2176).

secreted by the CD45RB$^+$ Rh123$^-$ cells late during culture (72 h). On the other hand, CD45RB$^-$ Rh123$^-$ (memory) cells secreted high titers of IFN-γ as expected.

Lymphokine gene expression in sorted CD4$^+$ subpopulations was also analyzed by reverse PCR 48 h after induction with anti-CD3 mAb (Fig. 9). In agreement with the supernatant results, CD45RB$^+$ Rh123$^+$ cells expressed IL-2 mRNA only, while CD45RB$^+$ Rh123$^-$ cells expressed both IL-2 and IFN-γ mRNA. CD45RB$^-$ (memory) cells on the other hand expressed both IL-4 and IL-10 mRNA in addition to IL-2 and IFN-γ. Neither IL-5 nor IL-6 mRNA could be detected at significant levels in these experiments.

4 Discussion

The data presented here confirm and extend other recent reports [18–23] indicating that efflux of the fluorescent dye Rh123 in leukocytes correlates with functional activity of P-gly, the molecule responsible for multidrug resistance in tumor cells. In particular, Rh123 efflux in mouse lymphocytes was blocked by low temperature and by several compounds (verapamil, CsA and PSC 833) known to interfere with drug resistance mediated by P-gly. Most importantly, the hierarchy of inhibition of Rh123 efflux in

lymphocytes by these compounds (PSC 833 > CsA > verapamil) was similar on a molar basis to that shown previously for reversal of drug resistance in tumor cells [35, 36]. Taken together, the data suggest strongly that Rh123 efflux in lymphocytes is mediated primarily (if not exclusively) by P-gly.

A striking result of our analysis was the extreme heterogeneity in P-gly activity among mouse peripheral T cells. Whereas virtually all $CD8^+$ T cells extruded Rh123 rapidly (within 3 h), $CD4^+$ T cells extruded the dye at a slower rate and a significant fraction ($\sim 50\%$ of $CD4^+$ cells in adult mice) did not demonstrate any detectable P-gly activity using this assay. The reasons for such marked differences in P-gly function are unclear, but appear to be conserved in mouse and human peripheral T cell subsets, as evidenced by several recent reports [18–23]. In this context preliminary studies of Rh123 efflux in mouse thymocytes indicate that P-gly function is acquired relatively late during T cell development and can only be readily detected in the mature $CD8^+$ thymic subset (manuscript in preparation).

In considering the physiological relevance of the observed heterogeneity of P-gly function in T lymphocyte subsets, it is important to emphasize that the natural substrate(s) of P-gly *in vivo* has not yet been identified. If (as might be predicted from drug resistance studies in tumors) P-gly is normally involved in the removal of potentially toxic substances, it could be speculated that detoxification via P-gly is more important for $CD8^+$ cells (which produce toxic molecules in the course of performing their cytolytic effector function) than for $CD4^+$ cells. In this regard preliminary experiments indicate that blocking of P-gly activity by PSC 833 or verapamil does not directly interfere with cytolytic function. An alternative possibility (suggested by the fact that P-gly can serve as an ATP-dependent chloride channel [45]) is that P-gly may be involved in some metabolic function that is modulated during T lymphocyte development and activation. With the recent development of mutant mice disrupted in their mdr 1a or mdr 1b genes [46, 47], additional insight into the physiological basis of P-gly heterogeneity among T cells may be forthcoming.

The heterogeneity of P-gly function among $CD4^+$ peripheral T cells prompted a more detailed phenotypic analysis of these cells. Multiparameter flow microfluorometric analysis of Rh123 efflux versus a panel of activation/memory markers established that the $Rh123^+$ $CD4^+$ subset had a phenotype characteristic of resting naive cells (*i.e.*, $CD45RB^+$ $CD44^{lo}$, $CD62L^+$, $CD69^-$, $CD25^-$). Interestingly, the $Rh123^-$ $CD4^+$ population could be further subdivided into two major subsets based on CD45RB expression. In particular the $CD45RB^-$ $Rh123^-$ subset included $\sim 50\%$ cells with an activated/memory phenotype (*i.e.*, $CD44^{hi}$, $CD62L^-$, $CD69^+$, $CD25^+$). In contrast, $CD45RB^+$ $Rh123^-$ cells were identical in phenotype to $CD45RB^+$ $Rh123^+$ cells using the same panel of mAb. Taken together, these data indicate that P-gly function is a useful parameter for distinguishing a novel subset of "naive" $CD4^+$ cells which would not be distinguishable on the basis of commonly used activation/memory markers.

Further comparison of the effector function of sorted $Rh123^+$ and $Rh123^-$ subsets of $CD45RB^+$ $CD4^+$ T cells indicated that the $Rh123^-$ population proliferated better and secreted higher levels of IL-2 upon challenge with a variety of polyclonal stimuli including Con A, SEB, $Mls\text{-}1^a$, allogeneic cells and immobilized anti-CD3 mAb. Furthermore, this subset produced detectable amounts of IFN-γ (assessed both by bioassay and RT-PCR), whereas the $Rh123^+$ $CD45RB^+$ subset was negative for IFN-γ production. As expected from previous studies [1–6, 10–13], the $CD45RB^-$ (memory phenotype) subset produced the highest levels of IFN-γ as well as detectable amounts (by RT-PCR) of IL-4 and IL-10. Based on these functional data it appears that the $CD45RB^+$ $Rh123^-$ subset has a "preactivated" phenotype which is intermediate between truly naive ($CD45RB^+$ $Rh123^+$) and memory ($CD45RB^-$ $Rh123^-$) cells. Others have implicated mouse $CD45RA^-$ $CD4^+$ cells as being hyperreactive with respect to proliferation and IL-2 production upon TCR cross-linking [48]. However, it is difficult to compare these latter data with our own in view of the fact that CD45RA mAb do not clearly define a biphasic distribution of $CD4^+$ cells in the mouse ([48, 49] and our unpublished data).

The developmental relationship among the different cell subsets distinguished by Rh123 efflux and CD45RB phenotype are at this point unknown. In view of their hyperreactivity with respect to proliferation and IL-2 secretion $Rh123^-$ $CD45RB^+$ cells could represent an intermediate stage in the differentiation pathway between $Rh123^+$ $CD45RB^+$ naive cells and $Rh123^-$ $CD45RB^-$ memory type cells. According to this scenario the $Rh123^-$ $CD45RB^+$ population would represent a functionally primed/preactivated subset that has not yet undergone changes in expression of surface markers normally associated with activation.

An alternate possibility for the origin of $CD45RB^+$ $Rh123^-$ cells is that they represent a population of "disguised" activated cells that have regained CD45RB expression. In this model, antigenic stimulation of naive $CD4^+$ T cells would lead to rapid changes in expression of a variety of markers (induction of CD25 and CD69, down-regulation of CD45RB and CD62L) accompanied by up-regulation of P-gly activity. Following this stage activation markers could theoretically revert to their initial state in the absence of further stimulation. Indeed CD25 and CD69 are known to be transiently expressed following T cell activation, and several studies support the idea that changes in the expression of CD45 isoforms and CD62L are also reversible [50–52]. If the up-regulation of P-gly activity was, however, not reversible, such a series of events would lead to accumulation of $Rh123^-$ cells with an otherwise naive phenotype. This model is consistent with the preferential accumulation of $CD45RB^+$ $Rh123^-$ cells in aged mice as well as with the presence of acutely activated ($CD25^+$ $CD69^+$) cells exclusively in the $CD45RB^-$ $Rh123^-$ subset. Further analysis of the kinetics of appearance of $Rh123^-$ cells following superantigen activation in normal mice or specific antigenic stimulation in TCR transgenic mice should help to elucidate the differentiation pathway leading from naive to memory T cells.

Finally, it is worth noting that the variations in P-gly function observed here and elsewhere [18–23] for subsets of peripheral T cells may have clinical implications. In this context it is theoretically possible that the multidrug resistance properties of lymphomas of T cell origin may

reflect the constitutive P-gly activity of the subsets from which they are derived. A careful phenotypic analysis of T cell lymphomas from patients receiving chemotherapy might help to resolve this important issue.

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